Journal of Chromatography, 122 (1976) 73–84
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CHROM. 9073

APPLICATIONS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE PHARMACEUTICAL INDUSTRY

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SUMMARY

A survey of the literature has been carried out, covering pharmaceutical applications of high-performance liquid chromatography (HPLC) from 1972–1975. The abstracts are divided into seven groups, *viz. alkaloids, antibiotics, nitrogen-containing compounds, steroids, sulphur-containing compounds, formulations*, and general analytical techniques. A general resumé of the development of HPLC in our own laboratory from 1969 onwards is given together with fourteen chromatograms covering the spectrum of our more novel activities in pharmaceutical analysis.

INTRODUCTION

The 1968 Medicines Act legislation in Great Britain, together with similar legislation in the rest of the world, led to a further tightening of the screws on the production and control of pharmaceuticals. More searching questions than ever before began to be asked about the control of impurities in starting materials, intermediates, and bulk medicinals. As a direct result of this, analysts in the pharmaceutical industry had to search for more specific and also more sensitive methods of analysis than ever before. They turned to: gas–liquid chromatography (GLC), thin-layer chromatography (TLC) and liquid chromatography (LC). TLC and LC were not readily acceptable because of the difficulty in quantifying TLC and because of the slow and inefficient nature of LC. It is therefore not surprising that GLC bore the main brunt of the burden of increase in sample numbers; and every conceivable method was adopted in order to make these complex pharmaceutical molecules volatile. However, with certain groups of compounds, *e.g.*, corticosteroids[1] and antibiotics[2], the use of GLC was impossible and one had to resort entirely to LC. Figs. 1 and 2 illustrate typical chromatograms obtained by the latter method, clearly demonstrating its inefficiency and slowness.

Mid 1969 saw the import of the first commercial liquid chromatographs from the United States and, after the first chromatogram of a steroid mixture had been run, the potential of the technique in pharmaceutical analysis was understood. The dilemma analysts found themselves in at this stage was that the only instruments available were the large research type, costing £10,000–12,000, so when the first

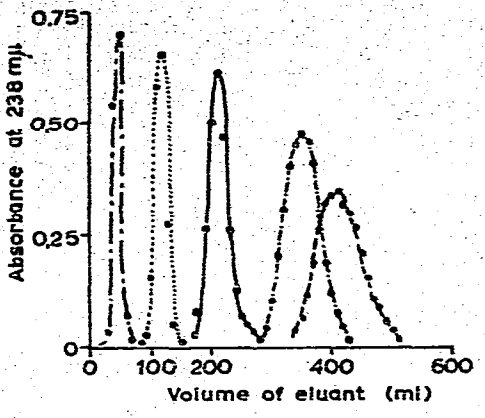


Fig. 1. Curves relating absorbance volume for fluocinolone acetonide and other currently available corticosteroids. ———, Betamethasone 17-valerate; ·····, hydrocortisone acetate; ———, prednisolone acetate; –·–·–·, triamcinolone acetonide; ———, fluocinolone acetonide.

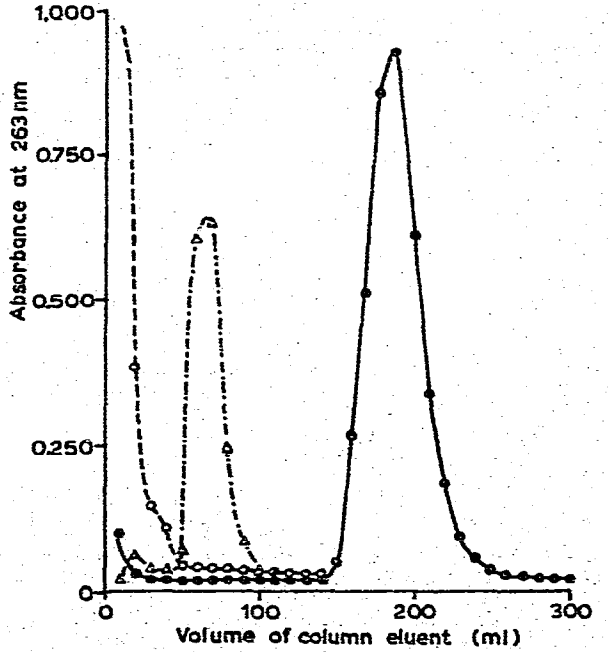


Fig. 2. Graph relating absorbance and retention volume for oxytetracycline and possible impurities. ○—○, β-apooxytetracycline; △–·–△, chlortetracycline; ●—●, oxytetracycline.

instrument they used became saturated with samples they were unable to afford a second. It was well into 1972 before the "cheaper" instruments became available. This may be the reason for the slow take off in high-performance liquid chromatography (HPLC) and the relatively small amount of published data (less than 100 papers in six years), which led the first reviewer[3] in the pharmaceuticals field to observe: "To date a relatively limited number of practical applications of HPLC has been reported in

the literature. This paucity of publications can be expected to change very rapidly with the ever increasing availability of commercial instrumentation, with improved column technology and with the realisation that many separations which had heretofore been both tedious and complicated, may now be effected quite readily by means of this powerful technique". More surprising observations from our own point of view are: (1) The very small number of contributions originating from Great Britain, *i.e.* 12%. (2) The very small number of papers concerned with quantitative analysis — which report such important parameters to the analyst as reproducibility, standard error, etc. (3) The even smaller number of publications (less than 5%) recording variable-wavelength monitors for use at wavelengths other than 254 nm and the utilisation of the benefits which this presents of increased specificity and sensitivity and in many cases the added bonus of a reduction of interference from excipients.

PHARMACEUTICAL APPLICATIONS

Alkaloids

The applicability of HPLC for the analysis of alkaloids is probably one of the best recorded; about sixteen papers have been published so far[4–19], varying in their applicability from the analysis of plant extracts to the quantitative analysis of multi-component drug products. The columns used appear almost equally divided between pellicular packings, *e.g.*, Zipax and Corasil, and ion exchangers, *e.g.* SCX, WAX, and modified polystyrenes.

Antibiotics

The work in this field has been dominated by the examination of tetracycline and its derivatives. Antibiotics are produced by fermentation processes —most of the standard methods of analysis are microbiological. These procedures are useful for measuring activity against a specific test organism but do not tell the analyst anything about the chemical purity of the medicinal. On the basis of these tests it is difficult to carry out any process development work to increase the purity or yield of the product. Nine papers have so far been published[20–28], using a wide variety of adsorbants, *e.g.*, Kieselguhr, ion exchangers, and pellicular packings, *e.g.*, Zipax, HCP, and Bondapak C_{18}, but almost exclusively eluting with some form of buffer system.

Nitrogen-containing compounds

This should be a popular area for exploitation in pharmaceutical analysis. Contained in this group are the tranquillisers: benzodiazepines, butyrophenones, carbamates, phenothiazines, thioxanthenes, etc. Because of the basic nature of many of the nitrogen-containing compounds, eluant phases in this group often contain bases, *e.g.*, ammonia, diethylamine; the complete range of column packings are recorded from ion exchangers through pellicular packings to adsorbants. Nineteen references to separations involving nitrogen-containing compounds have been found[29–47].

Steroids

This was really the area where the true potential of HPLC in pharmaceutical analysis was first seen. Derivatisation followed by GLC was extremely difficult for

this class of compounds in general and impossible for corticosteroids in particular. Steroids are, in general, potent compounds and as such are formulated in low-dosage formulations, hence the analyst is not only faced with an involatile compound but with only a very small amount of it in a formulation, usually a tablet, a cream, or an ointment. Undoubtedly the best way to tackle a cream or ointment is by HPLC. Much of the early work in this field was carried out on pellicular columns usually with reversed-phase eluants. Of late the pattern has been changing to adsorbants with organic eluant phases. Eleven references[48–58] are given to work carried out in this field to date.

Sulphur-containing compounds

This is an important group of compounds, containing the sulphonanides. Most of the published separations[59–66] are on ion exchangers with buffer solution eluants.

Formulations

HPLC really makes its greatest contribution in this area. Because the complete analysis is carried out in the liquid phase —either aqueous or non-aqueous— the extraction of the desired constituent from a formulation can be developed in such a way that the extracted material is miscible with the eluant. Because of the sensitivity of the technique, smaller amounts of sample may be taken, which in turn reduces the interference from excipients. In many cases it is sufficient to grind tablets with methanol, filter, and apply an aliquot of the extract to the column. With ointments it is often sufficient to shake the formulation with a mixture of aqueous alcohol and isooctane in order to remove the fatty excipients from the drug. It is surprising on reviewing the literature to find so few references to the analysis of formulations[67–76].

General techniques

This section covers the whole field of pharmaceutical analysis, from a general review paper on applications[77–82], drug purity profiles[78], to an excellent paper[81] on quick identification methods of accurate stability for active ingredients in analgesics. This paper is one of the few recorded which quote quantitative data comparing peak height and peak area measurements, and also tables on accuracy. Five papers were found[85–89] which discuss the use of fluorescence detectors, with special reference to pharmaceutical analysis. Two general papers were published studying columns and eluant systems; the first, by Parris[90], describes the use of ternary liquid systems containing dichloromethane, methanol, and water on the adsorbant Zorbax-Sil. The second, by Twitchett[91], evaluates the use of octadecylsilane chemically bonded to 10-μm silica (Bondapak C_{18}). The drugs selected are eluted with aqueous methanol at different concentrations. Retention values for 30 compounds are given. A review paper by Frei[92] discusses instrumental needs in pharmaceutical analysis, and illustrates this with applications to the separation of alkaloids, vitamins, antibiotics, barbiturates, and glycosides. The last paper describes the sophistication of coupling a liquid chromatograph to a mass spectrometer[79] and illustrates its application to the separation and identification of three sulphonamides.

In Great Britain since 1969 the development of the technique of LC has been taking place in two distinct phases. 1969–1973 saw much instrument development in

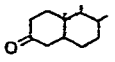
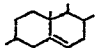
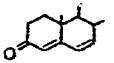
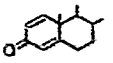
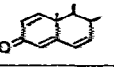
the two main areas of pumps and detectors. Pumps varied in complexity and reliability and the interminable argument on constant pressure *versus* constant flow has been going on to this day. Detectors are really the problem area in that we have no universal detector. The detectors which have claimed to be universal are not sensitive enough and have not stood the test of time when compared to the UV and refractive index detectors. In the pharmaceutical industry we are fortunate in that the majority of compounds produced have some kind of UV absorbance, and if the extinction coefficient is as low as 20 at 220 nm, quantitative analyses can still be carried out using the variable-wavelength detector, which was introduced in early 1972.

Table I clearly shows the variation in absorbance maxima in steroids. The ability to select the monitoring wavelength offers the following advantages: (1) greater sensitivity, (2) greater specificity, especially if the impurities or degradation products do not absorb at that wavelength, and (3) in many cases a reduction of interference from excipients.

The development of new instruments seemed to lose impetus from 1973 onwards and in recent years the effort has been on the development of column technology. For the first two or three years HPLC was carried out almost exclusively on pellicular materials, from 1973 onwards the fashion has been to turn to adsorbants, especially

TABLE I

ULTRAVIOLET ABSORPTION OF CHROMOPHORIC GROUPS IN STEROIDS

Chromophores	*Ultraviolet absorption*				
	λ_{max} *(nm)*	ε_{max}	*Example*	λ_{max} *(nm)*	ε_{max}
Carbonyl	170–200 280–300	5,000–10,000 50– 100		282	31
Double bond	180–225	500– 5,000		204	3,300
α,β-Unsaturated ketone	230–270	10,000–18,000		253	11,200
Conjugated dienes (different rings)	220–260	14,000–28,000		232 239 248	21,500 23,500 16,000
Conjugated dienes (same ring)	250–285	5,000–15,000		262 271 282 293	7,700 11,400 11,900 6,900
Conjugated polyenes	280–350	5,000–20,000		306	14,500
Diamones (different rings)	280–300	10,000–30,000		284	28,000
Diamones (same ring)	240–315	5,000–15,000		244 223	15,000 13,500
Triamones	220–380	10,000–30,000		256 298	11,900 15,300

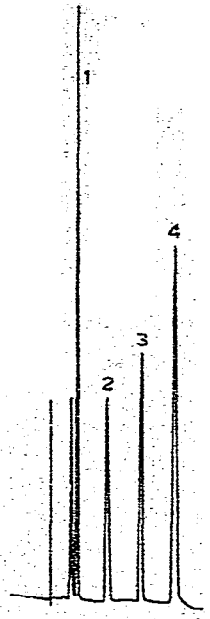


Fig. 3. Chromatogram of a test mixture for checking the efficiency of home-packed microparticulate columns. Column, 10 cm × 4.6 mm I.D., packed with 5-μm silica gel; solvent system, *n*-hexane–1 % acetonitrile; pressure, 100 p.s.i.; flow-rate, 1 ml/min; attenuation, 0.5 a.u.f.s. 1 = Phenanthrene; 2 = acetophenone; 3 = nitrobenzene; 4 = 2,4-dinitrotoluene. Wavelength, 254 nm.

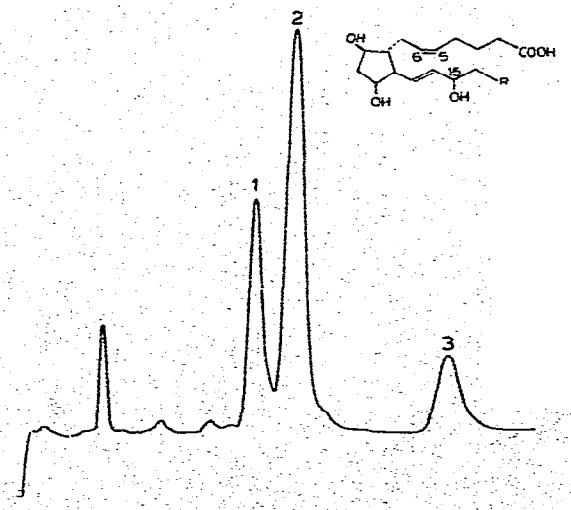


Fig. 4. Chromatogram of a synthetic prostaglandin mixture. Column, (25 cm × 2.2 mm I.D.) packed with Zorbax-Sil; pressure, 800 p.s.i.; flow-rate, 0.5 ml/min; attenuation, 0.2 a.u.f.s.; solvent system, *n*-hexane–ethanol–acetic acid (475:25:0.5); wavelength, 220 nm. 1 = 15-Epiprostaglandin; 2 = prostaglandin; 3 = 5,6-trans prostaglandin.

silica, as graded grains became available and slurry-packing techniques were developed. Columns of 6000–8000 theoretical plates for 10-cm columns have now become commonplace. We now find ourselves going through the whole ritual again of permanently bonded stationary phases (octadecylsilane ion exchangers, etc.) but this time onto silica rather than the pellicular packings. Chromatograms involving small microparticulate columns and variable-wavelength detection are shown in Figs. 3–7.

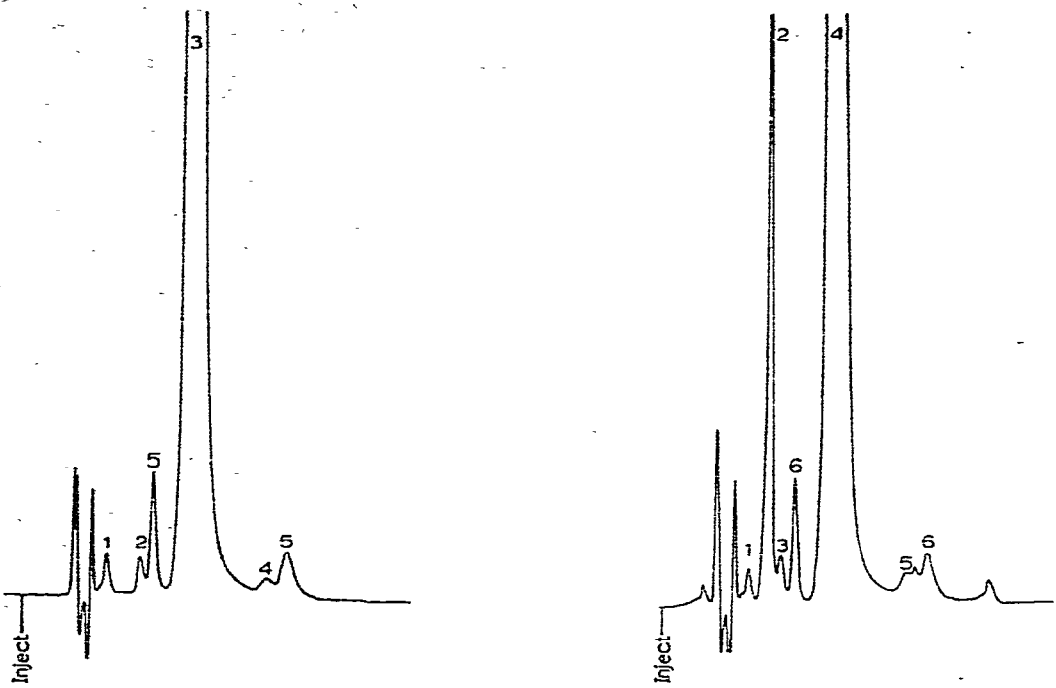


Fig. 5. Chromatogram of oxytetracycline bulk drug. Column, (20 cm × 4.6 mm I.D.) packed with 5-μm Lichrosorb SI-60; solvent system, 0.03 *M* EDTA disodium salt + 0.03 *M* KH_2PO_4 + 40% methanol (pH adjusted to 5.7 with 0.1 *M* NaOH); pressure, 1500 p.s.i.; flow-rate, 0.6 ml/min; attenuation, 0.2 a.u.f.s.; wavelength, 263 nm. 1 = β-Apooxytetracycline; 2 = α-apooxytetracycline; 3 = oxytetracycline; 4 = anhydrooxytetracycline; 5 = unknown.

Fig. 6. Chromatogram of oxytetracycline showing the inclusion of an internal standard, sulphamethazine. Column, (20 cm × 4.6 mm I.D.) packed with 5-μm Lichrosorb SI-60; solvent system, 0.03 *M* EDTA disodium salt + 0.03 *M* KH_2PO_4 + 40% methanol (pH adjusted to 5.7 with 0.1 *M* NaOH); pressure, 1500 p.s.i.; flow-rate, 0.6 ml/min; attenuation, 0.2 a.u.f.s.; wavelength, 263 nm. 1 = β-Apooxytetracycline; 2 = sulphamezathine (internal standard); 3 = α-apooxytetracycline; 4 = oxytetracycline; 5 = anhydrooxytetracycline; 6 = unknown.

Fig. 7 shows the difference in sensitivity of the impurities in this mixture when monitored at 254 nm rather than 273 nm. Fig. 8 demonstrates the usefullness of a double-beam spectrophotometer with low-volume flow cells for "stop–flow" spectra of peaks. The chromatogram is run as normal. When an unidentified peak appears on the chart the flow is "stopped at the top of the peak" and the spectrum of the trapped fraction scanned. This information is invaluable when running a formulated sample

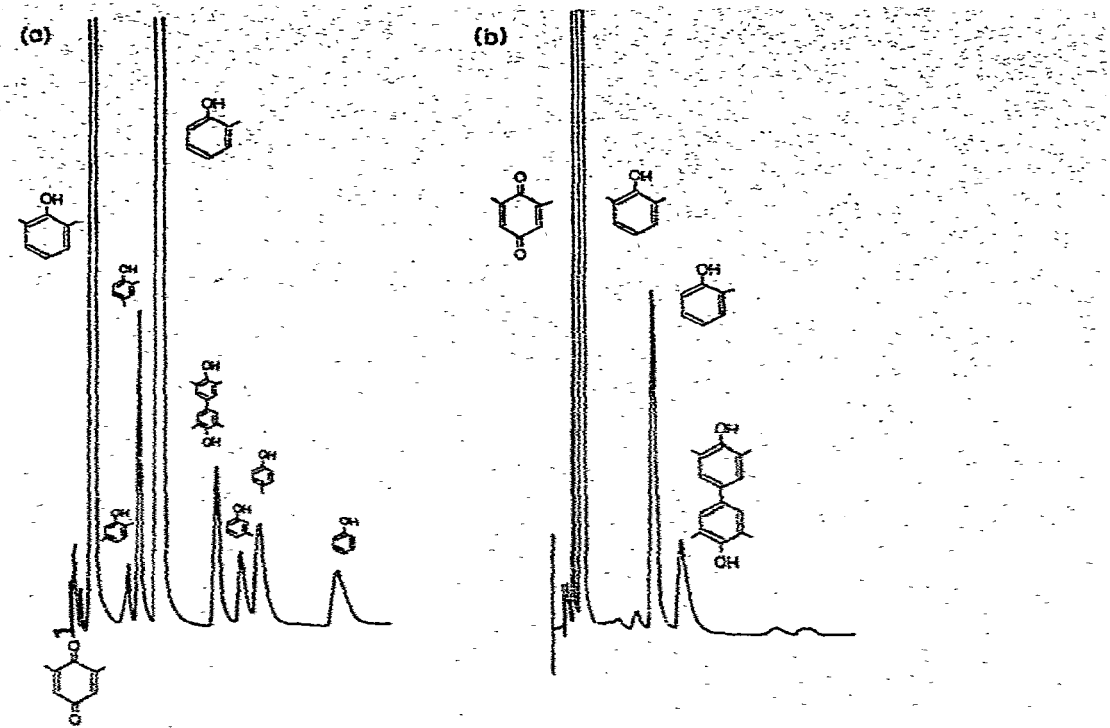


Fig. 7. Chromatograms of substituted phenolic mixtures demonstrating the difference in response at 254 and 273 nm. (a) Column, 10 cm × 4.6 mm I.D., packed with 5-μm Spherisorb S5W silica; pressure, 150 p.s.i.; attenuation, 0.05 a.u.f.s.; flow-rate, 2 ml/min; solvent system, *n*-hexane + 1% acetonitrile + 0.2% ethanol; wavelength, 273 nm. (b) Same conditions as (a), except wavelength, 254 nm.

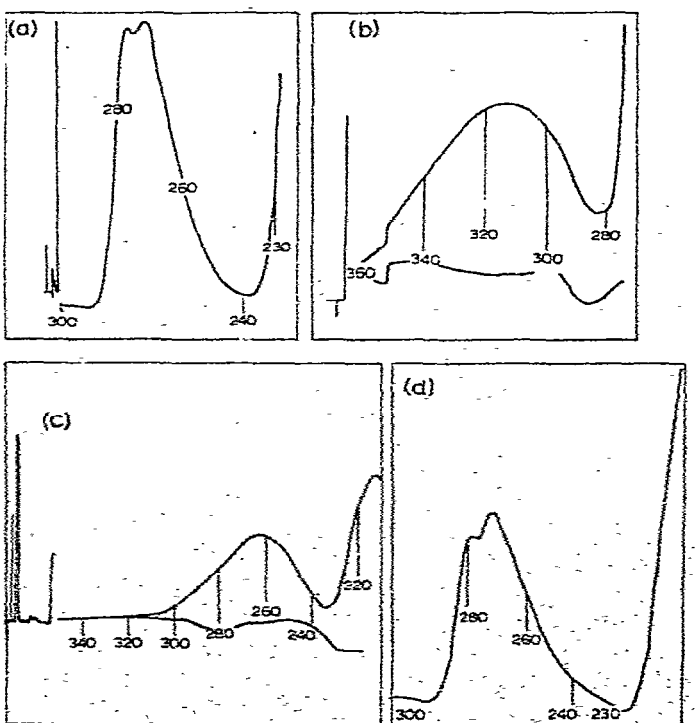


Fig. 8. Spectra of peaks trapped in the flow cell of a double-beam spectrophotometer by the "stop-flow" technique. (a) Peak (1), 2,6-disubstituted phenol; (b) quinone; (c) dimer; (d) peak (3), 2-sub-

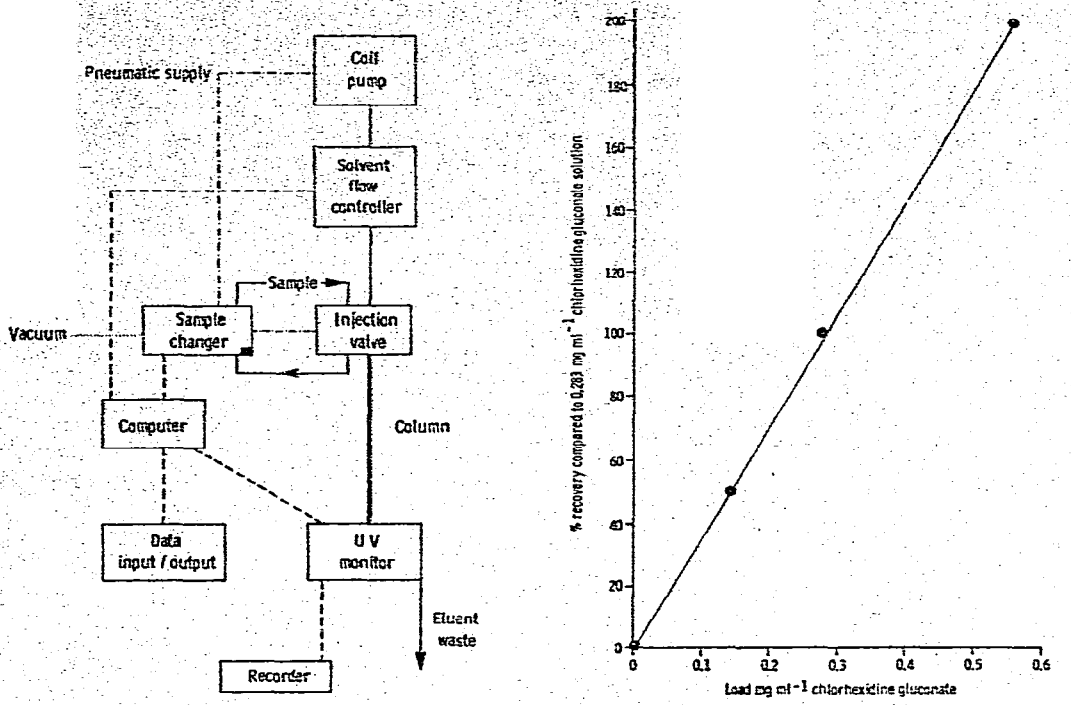


Fig. 9. Schematic diagram of an automated high-pressure liquid chromatograph. — — —, Signal and computer lines; · · · · · ·, vacuum lines; —·—·—·, pneumatic lines; ———, hydraulic lines.

Fig. 10. Graph demonstrating linearity of chromatograph response to chlorhexidine.

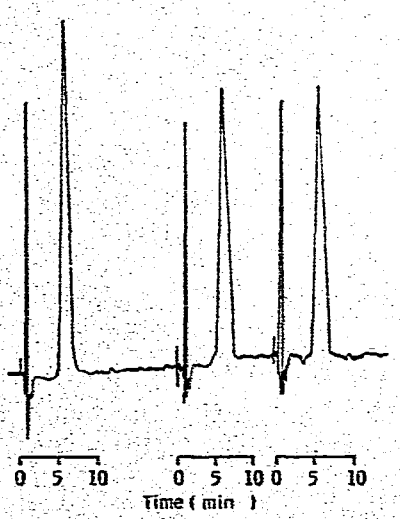


Fig. 11. Chromatograms of chlorhexidine standard (left) and duplicate injections of sample from a patch testing kit (middle and right). First peaks are due to the solvent. Column (10 cm × 4.6 mm I.D.) packed with 11 μm silica gel (Partisil); pressure, 300 p.s.i.; flow-rate, 1 ml/min; attenuation, 0.1 a.u.f.s.; wave length, 254 nm; solvent system, 0.02 *N* acetonitrile.

extract or a degraded sample for the first time. In many cases it will tell you if the peak obtained is from the active agent or from the excipients. As stated previously in this paper, when a method has been developed for a new pharmaceutical, and an assay time of 10–15 min has been achieved, samples appear from all directions —process development, stability, formulation development— and very soon the number exceeds that which is humanly possible to be analysed by one person during a 7½-h day. The machine then has to be made to work overnight, using automated injection and some form of data handling. This procedure has been carried out for the analysis of chlorhexidine[93]. The schematic system is shown in Fig. 9 and the results obtained are shown in Figs. 10–12.

Fig. 12. Chromatograms of duplicate injections of chlorhexidine standard (left) and an extract of Savlon baby lotion (right). Column (10 cm × 4.6 mm I.D.) packed with 11-μm silica gel; solvent system, acetonitrile–0.02 *N* sulphuric acid in water (91.5:8.5); pressure, 300 p.s.i.; flow-rate, 1 ml/min; attenuation, 0.1 a.u.f.s.; wavelength, 254 nm. First peaks are due to solvent or solvent and excipients.

CONCLUSION

If asked to predict the progress over the next five years, we would suggest a continuation of our present course, *i.e.* development of more permanently bonded stationary phases on silica and alumina, a more general acceptance of variable wavelength, and an appreciation of the contribution of UV spectra. Because of the high cost of capital equipment more mileage must be obtained out of existing machines through automated injection and data handling.

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